Mol Biol Rep (2013) 40:2205–2213
DOI 10.1007/s11033-012-2282-8

Oleylamine-carbonyl-valinol inhibits auto-phosphorylation activity of native and T315I mutated Bcr-Abl, and exhibits selectivity towards oncogenic Bcr-Abl in SupB15 ALL cell lines

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Received: 18 August 2012 / Accepted: 19 November 2012 / Published online: 5 December 2012

Abstract Chronic myeloid leukemia (CML) is characterized by the presence of $p210^{Bcr\text{-}Abl}$ which exhibits an abnormal kinase activity. Selective Abl kinase inhibitors have been successfully established for the treatment of CML. Despite high rates of clinical response, CML patients can develop resistance against these kinase inhibitors mainly due to point mutations within the Abl protein kinase domain. Previously, we have identified oleic acid as the active component in the mushroom *Daedalea gibbosa* that inhibited the kinase activity of Bcr-Abl. Here, we report that the oleyl amine derivatives, *S*-1-(1-Hydroxymethyl-2-methyl-propyl)-3-octadec-9-enyl-urea [oleylaminocarbonyl-*L*-N-valinol,oroleylaminocarbonyl-S-2-isopropyl-N-ethanolamine, oleylamine-carbonyl-*L*-valinol] **(cpd 6)** and *R*-1-(1-Hydroxymethyl-2-methyl-propyl)-3-octadec-9-enyl-urea [oleylamineocarbonyl-*D*-N-valinol, oleylaminocarbonyl-R-2-isopropyl-N-ethanolamine, or oleylamine-carbonyl-*D*-valinol] **(cpd 7)**, inhibited the activity of the native and T315I mutated Bcr-Abl. Furthermore, **cpd 6 and 7** exhibited higher activity towards the oncogenic Bcr-Abl in comparison to native c-Abl in SupB15 Ph-positive ALL cell line.

Keywords Oleylamine · L-/D-Valinol · Anti-tumor activity · Oleic acid · Bcr-Abl · Kinase · CML

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Introduction

CML is a clonal myeloproliferative disorder of the hematopoietic stem cells and accounts for 15 % of leukemias in adults [1, 2]. The translocation-related (9;22)-protein product is the Bcr-Abl fusion protein, which displays a constitutive tyrosine kinase activity, leading to the induction of aberrant proliferation and neoplastic transformation by constitutive activation of target signaling pathways. The Bcr-Abl induced phenotype in hematopoietic cells is characterized by an increased proliferation as well as by a reduced susceptibility to a variety of pro-apoptotic stimuli, including growth factor deprivation [3].

Imatinib (Gleevec), a selective inhibitor of Abl kinase is approved for the treatment of chronic phase CML (CP) and is included in most clinical trials for the treatment of Philadelphia-Chromosome positive (Ph+) acute lymphatic leukemia (ALL) [4–6]. Unfortunately, the clinical efficacy of the treatment with Imatinib continuously decreases with the advancement of the disease. Blast crisis CML or Ph+

ALL patients benefit from the treatment with tyrosine kinase inhibitors only temporarily or not at all [7]. Acquired resistance is mostly due to the acquisition of point mutations in Bcr-Abl by either reducing the affinity of the kinase domain for this ATP-competitor, or stabilizing the active conformation of the Abl kinase domain.

Second generation inhibitors of Abl kinase activity consists of compounds that can overcome Imatinib resistance. Nilotinib (Tasigna) is a novel ATP-competitive and is effective against a variety of cell lines expressing I-matinib-resistant Abl mutants, Dasatinib (Sprycel) is a dual Src/Abl kinase inhibitor that inhibits kinase activity of 14 out of 15 Imatinib resistant Abl mutants [8].

The major therapeutic challenge in Ph+ leukemia remains the "gatekeeper" mutation, T315I, which confers a global resistance against nearly all molecular therapy approaches targeting Bcr-Abl, such as ATP-competition, oligomerization inhibition or allosteric inhibition [9, 10]. Recently, Ponatinib, a multi-targeted tyrosine-kinase inhibitor, that is also effective against native Bcr-Abl and other Bcr-Abl kinase mutations, including T315I mutation, was approved by the FDA for the treatment of CML [11–13].

Previously, we identified a mycelium organic extract from the medicinal mushroom *Daedalea gibbosa* with selective anti-proliferating and apoptosis-inducing activities against the CML-derived K562 cells [14]. In addition, we identified an active fraction capable of directly inhibiting the kinase activity of Abl, as well as the auto-phosphorylation activity of native Bcr-Abl and the mutated forms, including the T315I mutation. The active fraction of *D. gibbosa* also inhibits the clonigenicity of native Bcr-Abl and its mutants when expressed in BaF3 cells [15]. Structure elucidation of the active moiety revealed that oleic acid is responsible for the Bcr-Abl inhibition activity in the *D. gibbosa* extract [16].

In this study we evaluated the activity of two novel oleyl amine derivatives, Oleylaminocarbonyl *D*/L-valinol, and found that both are active in inhibiting cellular auto-phosphorylation of native and T315I mutated Bcr-Abl.

Materials and methods

Chemicals

Oleyl amine 99 % was purchased from GL. Biochem China, dicyclohexylcarbodiimide, N-hydroxysuucinimide, sodium sulfate (Na_2SO_4), dimethylformamimde, dichloromethane, ethyl acetate, triethylamine, methanol (MeOH), ethanol (EtOH), hexane, chloroform (CHCl3) and pyridine were purchased from ACROS Chemicals Ltd (Israel). (NaOH), tetra-hydrofuran, dioxane and sulfuric acid (H2SO4) were purchased from Sigma-Aldrich Chemicals Ltd (Israel). Chemicals were used without further purification. Silica gel (Silica gel 60 (0.040–0.063 mm), thin layer chromatography (TLC Silica gel 60 F254) sheets were all purchased from Merck Ltd. Deuterated solvents for NMR: D2O, CDCl3, DMSO-d_6 was purchased from ACROS Chemicals Ltd (Israel). ^{1}H-NMR and ^{13}C-NMR data were collected using Varian Unity Inova 500 MHz spectrometer equipped with a 5-mm switchable tube and data were processed using the VNMR software.

Cell lines and cell cultures

Ba/F3 cells expressing Bcr-Abl were previously described [15]. Ba/F3 expressing JAK2 V617F were received from Prof. Shai Izraeli (Functional Genomics and Leukemia Research Pediatric Hemato-Oncology and Cancer Research Center, Sheba Medical Center, Israel) and were grown in RPMI 1640 with 2 mM L-glutamine supplemented with 10 % fetal bovine serum. Penicillin at 100 U/ml and streptomycin at 100 μg/ml were added to the culture media. All cell lines were grown at 37 °C in a humidified atmosphere with 5 % CO2.

Cellular auto-phosphorylation of Bcr-Abl

Ba/F3 cells expressing the native Bcr-Abl protein (4×10^5 cells/ml) were treated with oleic acid derivatives, Abl kinase inhibitors, and DMSO for 1 h. Cells were collected, washed once with cold PBS, and lysed as indicated before. Cell lysate supernatants (40 μg protein) were resolved on 8 % SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and analyzed by immune-blotting with Anti-phospho-c-Abl(Tyr245) polyclonal antibody (Cell Signaling Technology, USA). The phosphorylated level of Bcr-Abl protein was compared to total Abl, which was detected using Anti-c-Abl monoclonal antibody (Santa Cruz Biotechnology, USA).

Trypan blue exclusion assay

Ba/F3 cells were plated (4×10^5cells/well) in six-well plates, with each well containing 3 ml medium. After 24 h, cells were treated with the appropriate agents. Solvent-treated samples were incubated with 1 % DMSO. Seventy-two hours later, the cells were collected, stained with 0.4 % trypan blue solution (1:1), and counted using a hemacytometer to determine IC_{50} values.

Colony-forming assay

A colony-forming assay was performed as previously described [15]. Briefly, cells (1×10^4) in 1 ml RPMI/10 % FBS medium were diluted in 1 ml of 0.6 % agar to give a final agar concentration of 0.3 % agar. The cell-agar mixture was poured on top of a hardened agar base in wells of

12-well plates and allowed to solidify. Once the top layer solidified, 1 ml of medium containing different treatments was placed on top to keep the agar moist. The cells were grown at 37 °C in a 5 % CO2 humidified atmosphere until colonies were visible (2–3 weeks). The plates were stained for 4 h with 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and the dye was extracted with 1 ml solubilization buffer (20 % sodium dodecyl sulfate [SDS], 50 % N,N-dimethyl-formamide, 25 mM HCL) for 24 h. The optical density was measured at 570 nm wavelength with a reference wavelength of 630 nm.

Cell proliferation (XTT) assay

The XTT method was used to evaluate the cytotoxicity of the oleic acid derivatives. Bcr-Abl positive cell lines (1.5×10^4) were maintained in the appropriate media using 96-well plates for 24 h, and then treated for an additional 72 h with the appropriate agents. A total of 50 µl of XTT solution were added to each well and incubated for three hours at 37 °C. The optical density was measured by a multi-well plate spectrophotometer at 405 nm. The concentrations inhibiting cell proliferation by 50 % (IC_{50}s) were calculated.

Synthesis of oleylamino-*L/D*-N-Valinol

Oleyl amine (0.8 g, 3 mmol) was dissolved in 10 ml dried pyridine. While stirred at room temperature (0.486 g, 3 mmol) carbonyldiimidazole were added. The mixture was stirred for 48 h. Reaction progress was monitored by TLC (100 % ethyl acetatae and I_2 as developer). When the starting material disappeared, one equivalent (3 mmol) of the desired aminoethanol *L*-Valinol or *D*-valinol was added and the mixture was stirred for 2 days at room temperature. Reaction progress was followed by TLC [100 % Ethyl acetate, I_2]. After the reaction termination, as was followed by TLC [100 % EtAc], the solvent was removed under reduced pressure and the resultant was purified employing column chromatography (100 % $CHCl_3$). The fractions containing the desired product were pooled, and volatiles were evaporated under reduced pressure.

Oleylaminocarbonyl-*L*-N-Valinol; [Oleylaminocarbonyl-*S*-2-isopropyl-N-ethanolamine] **(cpd 6):**

Yield = (1 g, 84.4 %), TLC (100 % CHCl3; I_2).

^{1}H-NMR (CDCl3, δppm): 0.87(t, 3H), 0.94(q, 6H), 1.28(m, 2H), 1.81(m, 2H), 2.01(m, 5H), 3.15(t, 2H), 3.51(m, 1H), 3.71(d, 2H), 5.34(dd, 2H).

^{13}C-NMR($CDCl_3$, δ ppm): 14.32, 18.98, 19.76, 22.90,27.15, 27.43,29.73, 29.94, 30.38, 32.13, 41.07,58.51, 65.43,130.10, 159.62.

Oleylaminocarbonyl-*D*-N-Valinol;[Oleylaminocarbonyl-*R*-2-isopropyl-N-ethanolamine] **(cpd 7)**

Yield = (0.830 g, 70.8 %). TLC (100 % CHCl3; I_2).

^{1}H-NMR(CDCl3, δppm): 0.87(t, 3H), 0.94(q, 6H), 1.28(m, 2H), 1.81(m, 2H), 2.01(m, 5H), 3.15(t, 2H), 3.51(m, 1H), 3.71(d, 2H), 5.34(dd, 2H).

^{13}C-NMR ($CDCl_3$, δppm):14.32,18.98,19.76,22.90,27.15, 27.43,29.73,29.94,30.38,32.13,41.07,58.51,65.43,130.10, 159.62.

Molecular docking

The crystal structures of Abl and JAK2 were retrieved from the Protein Data Bank (PDB) [17, 18] and docking to the two proteins was performed by the ADT (AutoDock Tools) program, an accessory program that allows the user to interact with AutoDock4.2 from a graphic user interface. Water molecules and other ligands were removed from the protein PDB file. Polar hydrogen atoms were added and Kollman united atomic charges assigned. The solvation parameters were added by the Addsol program (part of the ADT program) and the grid points were set to 80, 80, 80, the spacing value equivalent to 0.375 and the grid center to 16.32, 8.79, −35.89 for Bcr-Abl and 70, 70, 76, the spacing value equivalent to 0.375 and the grid center to −0.87, 2.66, 24.403 for JAK2. The structures of **cpd 6** and **cpd 7** were prepared using "CS ChemdrawPro" and "CS ChemBats3DPro" and optimized to their minimum energy. Ligand-docking was carried out with the AutoDock 4.2 Genetic Algorithm (GA) [19, 20].

Results

Previously, we identified oleic acid as the active component in the mushroom *D. Gibbosa* that inhibited the auto-phosphorylation and kinase activity of native and mutated Bcr-Abl, including the "gatekeeper" T315I mutation, which confers absolute resistance to Imatinib [14–16].

In this study, we examined the activity of a number of oleyl amine derivatives on Bcr-Abl auto-phosphorylation (data not shown). Figure 1 shows the synthesis and the structure of the two oleyl amine derivatives, Oleylamino-carbonyl-*L*-N-valinol **(cpd 6)** and Oleylaminocarbonyl-*D*-N-valinol **(cpd 7)** that were active in inhibiting Bcr-Abl auto-phosphorylation.

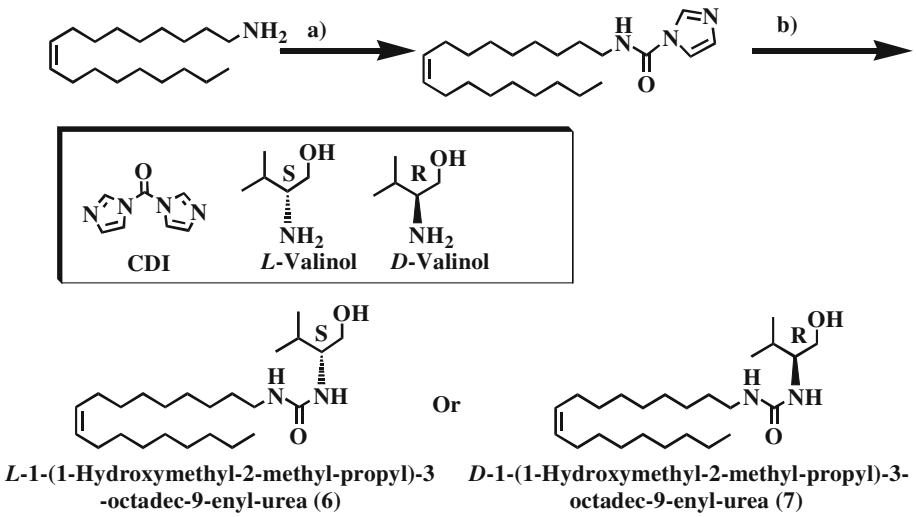

Fig. 1 Synthesis and chemical structure of oleylaminocarbonyl L-N-valinol [L-1-(1-Hydroxymethyl-2-methyl-propyl)-3-octadec-9-enyl-urea (cpd 6) and oleylaminocarbonyl D-N-valinol [D-1-(1-Hydroxymethyl-2-methyl-propyl)-3-octadec-9-enyl-urea] (cpd 7) as outlined in Materials and methods

Oleylaminocarbonyl-*D/L*-N-valinol inhibits native and mutated Bcr-Abl cellular auto-phosphorylation in Ba/F3 cells

The ability of the two oleyl amine derivatives, Oleylaminocarbonyl-*L*-N-valinol **(cpd 6)** and Oleylaminocarbonyl-*D*-N-valinol **(cpd 7),** to affect cellular Bcr-Abl auto-phosphorylation was monitored using immune-blotting.

Figure 2 shows that both derivatives inhibited the auto-phosphorylation activity of the native Bcr-Abl protein (Fig. 2a). In contrast to Imatinib, an ATP competitor, and GNF-2, an Abl allosteric inhibitor [10], the two derivatives also inhibited the auto-phosphorylation of the T315I mutated Bcr-Abl in a comparable potency to that exhibited against the native form (Fig. 2a). Furthermore, both oleyl amine derivatives were active in inhibiting the phosphorylation of STAT5α, a downstream Bcr-Abl target, from native and T315I mutated cells, in a comparable potency (Fig. 2a). Similarly, the two oleyl amine derivatives were also active in inhibiting the auto-phosphorylation of other Bcr-Abl mutations, such as the ATP binding loop mutants, Y253F (Fig. 2b). Interestingly, the activity of Oleylaminocarbonyl D/L-N-valinol against pBcr-Abl was much profound compared to their activity against pSTAT5α. Furthermore, **cpd 6** (Oleylaminocarbonyl-*L*-N-Valinol) exhibited an increased potency compared to **cpd 7** (Oleylaminocarbonyl-*D*-N-Valinol).

Effect of Oleylaminocarbonyl-*D/L*-N-valinol on Bcr-Abl cellular auto-phosphorylation in Ph+ patient-derived cell lines

Next, we tested the ability of the two Oleylaminocarbonyl D/L-N-valinol compounds to affect Bcr-Abl auto-phosphorylation using Ph+ patient-derived cell lines, such as K562, MEG-01, BV173, and SupB15. Results shown in Fig. 3 illustrated that derivative Oleylaminocarbonyl-*L*-N-Valinol **(cpd 6)** and derivative Oleylaminocarbonyl-*D*-N-Valinol **(cpd 7)** exhibited variable activity in inhibiting Abl phospho-tyrosine in the different patient-derived cell lines, as measured by pTyr Abl ELISA assay (Fig. 3a, b) [15].

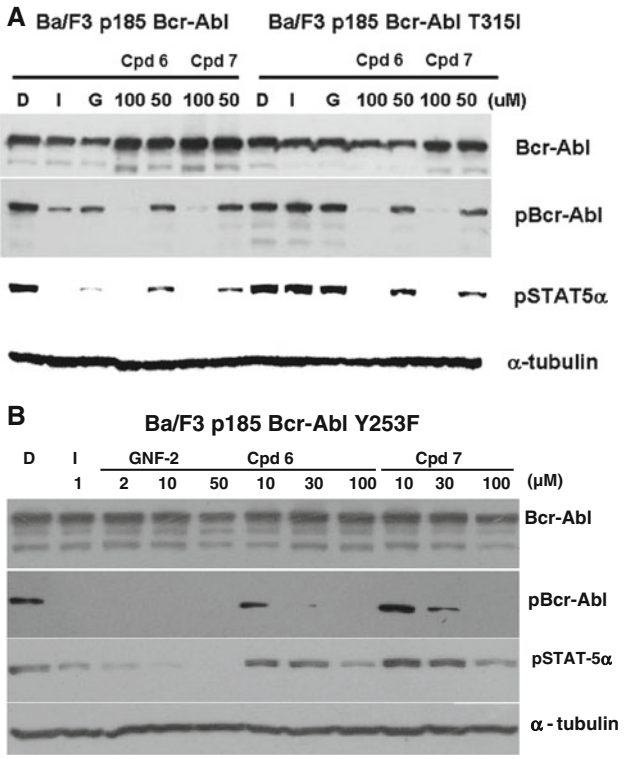

Fig. 2 Inhibition of pBcr-Abl and pSTAT5α in Ba/F3 cells carrying p185 Bcr-Abl, p185 Bcr-Abl T315I (**a**) and p185 Bcr-Abl Y253F (**b**) constructs by OleylaminocarbonylL-valinol **(cpd 6)** and Oleylaminocarbonyl D-N-valinol **(cpd 7)**. Immunoblots of Bcr-Abl, Abl, pBcr-Abl, pAbl and pSTAT5α in response to Oleylaminocarbonyl D-valinol and Oleylaminocarbonyl L-N-valinol were monitored. α-tubulin was used as loading control. *D*, DMSO, *I*, Imatinib at 1 μM, and *G*, GNF-2 at 10 μM

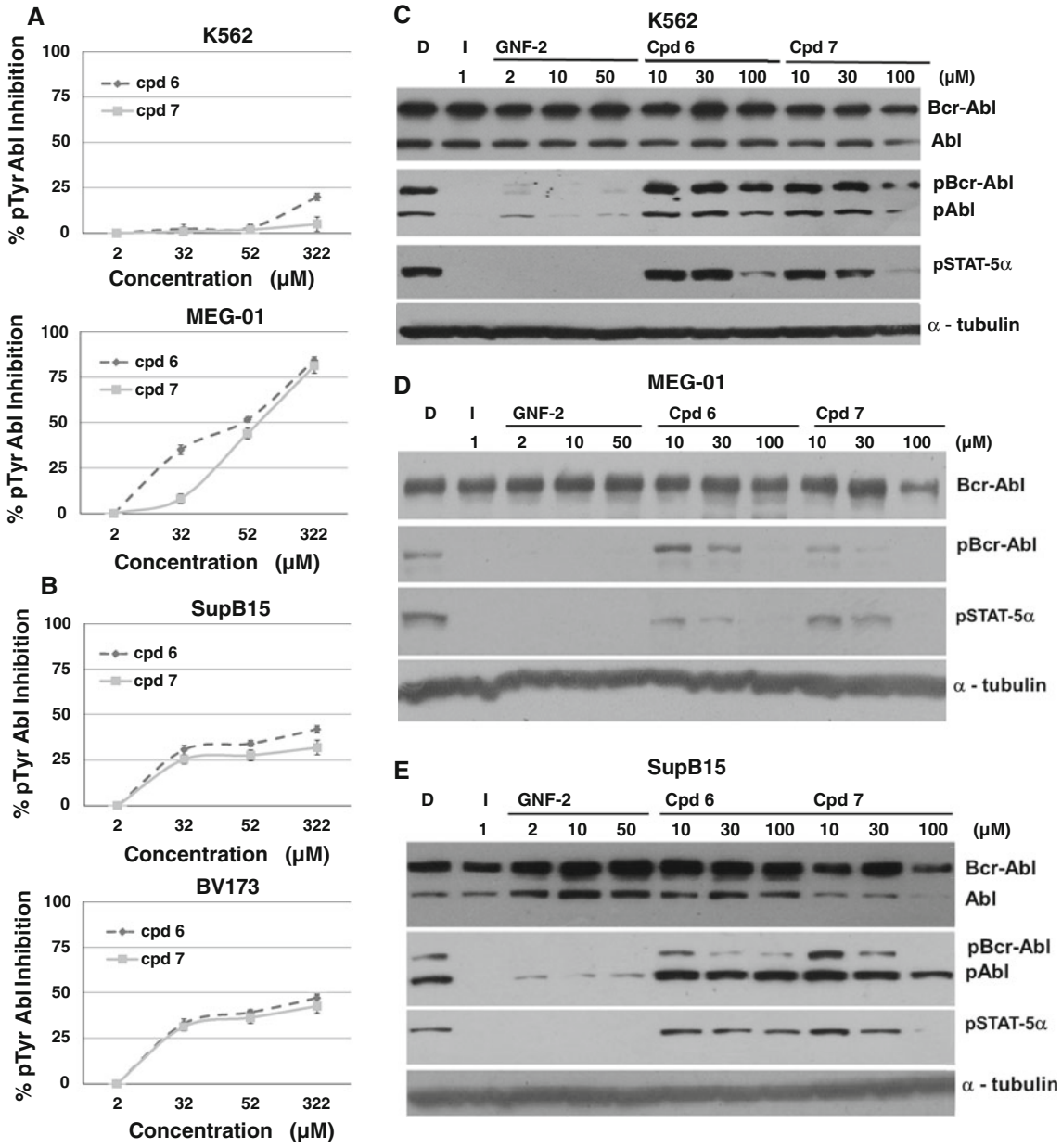

Fig. 3 Inhibition of Bcr-Abl auto-phosphorylation from ph+ patient-derived cell lines. Levels of Abl pTyrosine in response to Oleylaminocarbonyl D-N-valinol **(cpd 6)** and Oleylaminocarbonyl L-N-valinol **(cpd 7)** using K562 and MEG-01 (**a**) and ALL cell lines SupB15 and BV-173 (**b**). Immunoblots of Bcr-Abl, Abl, pBcr-Abl, pAbl and pSTAT5α in response to Oleylaminocarbonyl D-valinol and Oleylaminocarbonyl L-valinol were monitored in K562 (**c**), MEG-01 (**d**) and SupB15 (**e**) patient-derived cell lines. α-tubulin was used as loading control. *D*, DMSO and *I*, Imatinib

The two derivatives were effective in inhibiting Abl phospho-tyrosine levels in Ph+ megakaryoblastic MEG-01 cell lines (Fig. 3a). In contrast, they failed to inhibit Abl phospho-tyrosine levels in K562 cell lines (Fig. 3a), and showed only marginal activity in B-lineage precursor ALL SupB15 and BV173 cell lines (Fig. 3b).

Next, we followed the effect of the two oleyl amine derivatives on the levels of pBcr-Abl, pAbl and pSTAT5α in the various Ph+ patient-derived cell lines using immunoassay. Our data illustrated that while Bcr-Abl was expressed at comparable levels in the different Ph+ cell lines, the levels of the endogenous c-Abl varied significantly. Levels of endogenous c-Abl were low in MEG-01 (Fig. 3d) and Ba/F3 cells and significantly high in K562 (Fig. 3b) and SupB15 (Fig. 3c). Figs. 3c–e summarize the obtained results. Figure 3c shows that levels of pAbl and pBcr-Abl from K562 ($p^{210\ Bcr\text{-}Abl}$ CML) cell lines were significantly inhibited in the presence of Imatinib (1 μM) and GNF-2

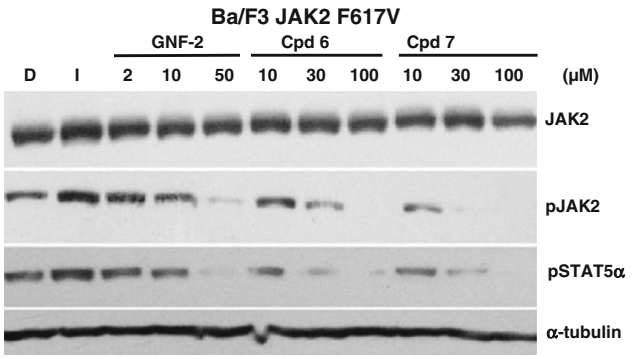

Fig. 4 Inhibition of V617F JAK2 phosphorylation by Oleylaminocarbonyl D/L-N-valinol compounds. Immunoblots of JAK2, pJAK2 and pSTAT5α in response to Oleylaminocarbonyl D-valinol and Oleylaminocarbonyl L-N-valinol were monitored. α-tubulin was used as loading control. *D*, DMSO and *I*, Imatinib

(2 μM–50 μM). However, the two derivatives were not effective in inhibiting neither pAbl nor pBcr-Abl (Fig. 3c). Similarly, levels of pSTAT5α were only marginally affected by the presence of the two derivatives at the highest concentration (100 μM). Treatment of MEG-01 cell lines ($p^{210\ Bcr\text{-}Abl}$ AMKL) with the two compounds resulted in a significant inhibition of pBcr-Abl and pSTAT5α (Fig. 3d). The two derivatives were also active in inhibiting pBcr-Abl levels in the SupB15 ($p^{190\ Bcr\text{-}Abl}$ ALL) cell line (Fig. 3e). Interestingly, **cpd 6** (Oleylaminocarbonyl-*L*-N-Valinol) exhibited a higher potency in inhibiting pBcr-Abl compared to **cpd 7** (Oleylaminocarbonyl-*D*-N-Valinol). In addition, levels of pSTAT5α were also affected by both compounds, with **cpd 6** showing higher potency compared to **cpd 7**. Interestingly, phosphorylation levels of endogenous c-Abl were only marginally affected by the presence of both derivatives in SupB15 cell lines (Fig. 3e).

Oleylaminocarbonyl D/L-N-valinol inhibits phosphorylation of JAK2 V617F

Previously, we showed that GNF-2, an Abl allosteric inhibitor, is moderately active in inhibiting auto-phosphorylation of V617F JAK2 kinase [24]. In this report, the ability of the two oleic acid derivatives to inhibit auto-phosphorylation of V617F JAK2 was monitored using Ba/F3 expressing V617F JAK2 kinase. Results, presented in Fig. 4, demonstrated that Imatinib (1 μM) was not active in inhibiting JAK2 phosphorylation. In contrast, GNF-2 at 50 μM was effective in inhibiting JAK2 phosphorylation as previously shown [24]. Similarly, the two oleic acid derivatives (**cpd 6** and **cpd 7**) significantly inhibited JAK2 phosphorylation at the highest concentration, and exhibited a partial inhibition at 30 μM. In addition, level of JAK2 downstream targets, such as pSTAT5α, was also inhibited by GNF-2 as well as by the two oleic acid derivatives, though with somewhat reduced potency in comparison to the observed effect on pJAK2 V617F.

Oleylaminocarbonyl D/L-N-valinol inhibits clonigenicity of cells harboring activated Bcr-Abl and JAK2 kinases

Anchorage-independent growth is one of the hallmarks of transformation, which is considered the most accurate and stringent in vitro assay for detecting malignant transformation of cells. Anchorage-independent growth, which approximates tumorgenesis of cancer cells, measures the proliferation and colony-formation of cancer cells in a semi solid culture media. Ba/F3 cells carrying either the native Bcr-Abl (p185), T315I mutated Bcr-Abl, and activated (V617F) JAK2 were utilized to monitor clonigenicity in the presence of Oleylaminocarbonyl D/L-N-valinol compounds. Results shown in Fig. 5 demonstrated that Imatinib, Nilotinib, and GNF-2 were active in inhibiting clonigenicity of Ba/F3 cells carrying the native Bcr-Abl, but not cells carrying the T315I mutation or activated JAK2. GNF-2 inhibited clonigenicity of cells carrying native and T315I Bcr-Abl with IC_{50} of 0.8 and 26 μM, respectively (data not shown). Presence of Oleylaminocarbonyl D-N-valinol and Oleylaminocarbonyl L-N-valinol were only moderately effective in inhibiting clonigenicity of Ba/F3 cells carrying the native Bcr-Abl, the T315I mutated Bcr-Abl, and the activated JAK2 constructs, with IC_{50} values of 69, 60, and 37 μM, respectively.

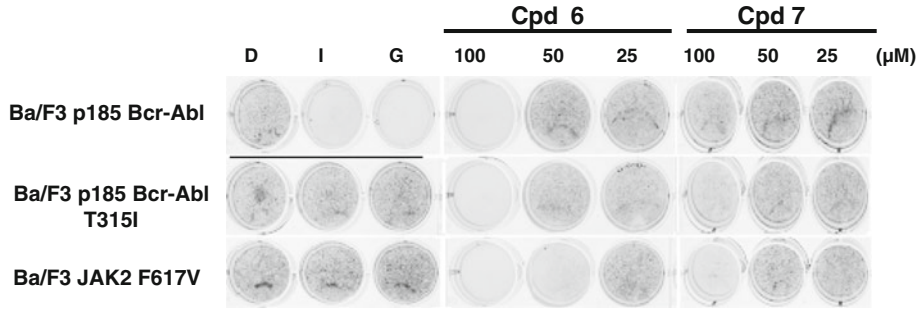

Fig. 5 Clonogenicity of Ba/F3 cells carrying native Bcr-Abl, T315I mutated Bcr-Abl, and V617F mutated JAK2. Cells grown on soft agar were treated with 0.5 % DMSO (*D*), Imatinib 1 μM (*I*), GNF-2 2 μM (*G*), cpd 6, and cpd 7 (25–100 μM)

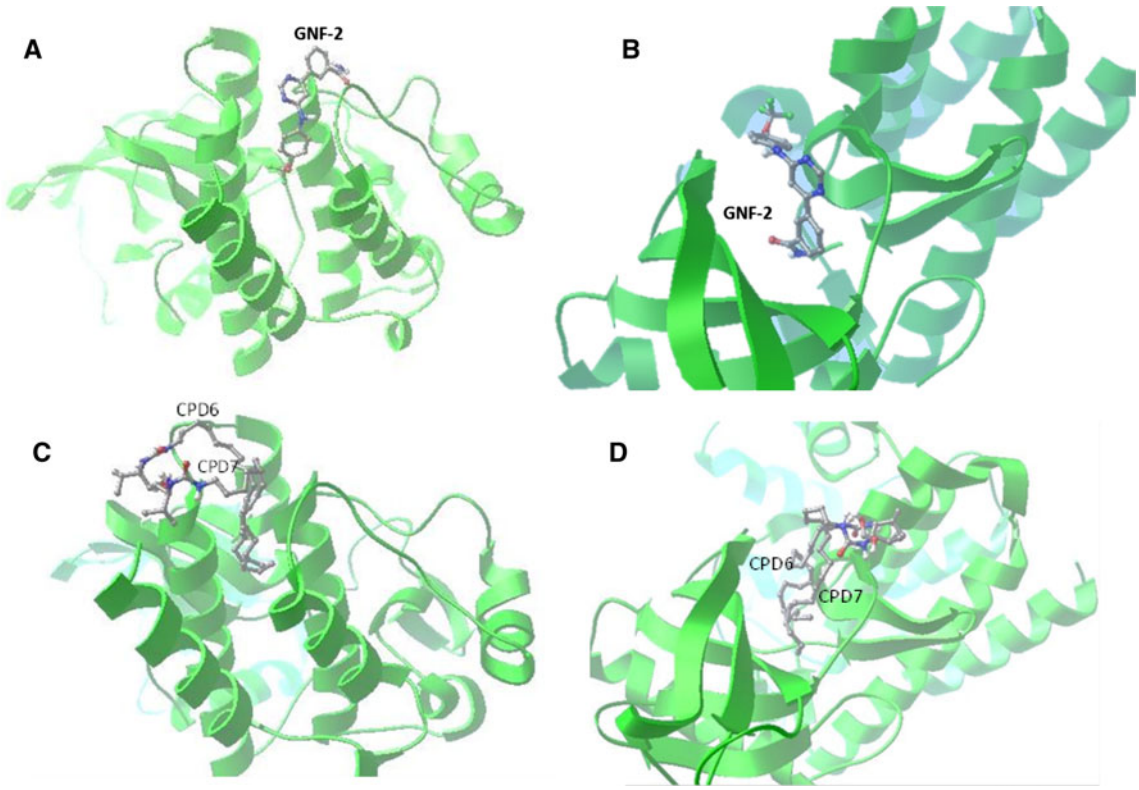

Fig. 6 Orientation of GNF-2, **cpd 6**, and **cpd 7** within Bcr-Abl (A, C) and JAK2 (B, D) at the myristoyl binding pocket. A and B show docking of GNF-2 to Abl (**a**) and JAK2 (**b**). Docking of **cpd 6** and **cpd 7** with Abl (**c**) and JAK2 (**d**) using autodock 4.2 program. The various ligands are oriented to the hydrophobic zones of the enzymes and interact with the hydrophobic myristoyl binding pocket in Abl. The binding energy of the interaction with Abl was −3.22 and −3.70 for **cpd 6** and **cpd 7**, respectively, and with JAK2 was −4.06 and −3.11 for **cpd 6** and **cpd 7**, respectively. The binding energy of the interaction between GNF-2 with Abl was −6.78, and −6.58 with JAK2

Discussion

In an attempt to discover a new chemical entity that will be effective in inhibiting the kinase activity of native and T315I mutated Bcr-Abl, we identified an active organic extract from the medicinal mushroom *D. gibbosa* [14], and in a subsequent study we identified the active component as oleic acid [16]. In the present study, we synthesized two oleyl amine derivatives; oleylaminocarbonyl L-N-valinol **(cpd 6)** and oleylaminocarbonyl *D*-N-valinol **(cpd 7)** and evaluated their activity against native and mutated Bcr-Abl.

The two oleylamine derivatives were active in inhibiting the auto-phosphorylation of native, T315I, and Y253F mutated Bcr-Abl. Moreover, both compounds inhibited the phosphorylation of STAT5α, a well-known Bcr-Abl downstream target. Interestingly, the Y253F mutated Bcr-Abl was more sensitive to the activity of the two oleylamine derivatives (Fig. 2b). Furthermore, the ability of the two compounds to inhibit the phosphorylation of STAT5α in Ba/F3 p185 Bcr-Abl Y253F cells was reduced in comparison to their ability to inhibit Bcr-Abl auto-phosphorylation in the same cells. Moreover, we noticed that the *L* isomer of oleylaminocarbonyl-L-N-valinol **(cpd 6)** exhibited enhanced activity in inhibiting Y253F Bcr-Abl auto-phosphorylation in comparison to the D isomer **(cpd 7)**, arguing that the L isomer of Oleylaminocarbonyl-D/L-N-valinol is somewhat more selective towards Y253F mutated Bcr-Abl.

In addition, we evaluated the activity of the two oleic acid derivatives against Bcr-Abl protein of different sizes (p185 Vs. p210), and against Bcr-Abl expressed in different cellular backgrounds. Our data illustrated that the two oleyl amine derivatives exhibited activity against $p185^{Bcr\text{-}Abl}$ produced in Ba/F3 $p185^{Bcr\text{-}Abl}$ cells and also in SupB15, as well as against the major product of Ph+ translocation, $p210^{Bcr\text{-}Abl}$ protein, such as in MEG-01 cell lines. However, the potency of the two derivatives seems to be influenced by the cellular background. While the two derivatives were potent in inhibiting $p210^{Bcr\text{-}Abl}$ in MEG-01, they showed only a marginal activity against $p210^{Bcr\text{-}Abl}$ in K562 cell line. The mechanism responsible for such a cell-type specificity remains to be elucidated.

Moreover, our results illustrated that the different Bcr-Abl positive cells exhibit different levels of the endogenous Abl protein (c-Abl) expression. While SupB15

expresses high levels of the endogenous c-Abl, a very minimal amount of c-Abl was observed in MEG-01. Furthermore, we observed that the two derivatives exhibited different potency towards the Abl proteins, endogenous c-Abl (myristoylated) and the oncogenic Bcr-Abl (non-myristoylated). The two oleyl amine derivatives were much more potent in inhibiting the non-myristoylated Bcr-Abl compared to the myristoylated endogenous c-Abl. Our findings are in agreement with an observation showing the ability of GNF-2 to inhibit the kinase activity of Bcr-Abl more potently than that of the myristoylated c-Abl by binding to the myristate-binding pocket in the C-lobe of the kinase domain [21]. Moreover, our findings are also in agreement with observations made by Choi et al. 2009, [21] and others, in which Brij-35, a non-ionic detergent consists of a polyoxyethylene group attached to a 12-carbon unsaturated moiety, commonly used in in vitro kinase assay, is speculated to act as a competitor for the binding to the myristate-binding pocket. Furthermore, our findings argue for the potential development of oncogenic-selective Abl inhibitors, similarly to the differential sensitivity of the oncogenic EGFR towards Erlotinib and Gefitinib compared to the wild-type EGFR receptor [22], and also to the differential sensitivity of the recently FDA approved vemurafenib (PL × 4032) towards activated BRAF V600E as compared to BRAF WT [23]. However, the bases for such selectivity of the two oleyl amine derivatives remain to be elucidated.

Although GNF-2 appears to be a selective Abl allosteric inhibitor, and exhibits no detectable inhibitory activity towards the closely related Src family of tyrosine kinases [21], our current findings demonstrated that GNF-2 also exhibited a significant inhibitory activity against native and activated JAK2 (personnel communication). In this study, our data illustrated (Fig. 4) that the two oleyl amine derivatives were also active in inhibiting the phosphorylation of V617F JAK2, and its downstream target, STAT5α, at a comparable potency as GNF-2.

As expected, the two oleyl amine derivatives were also active in inhibiting clonigenicity of Ba/F3 cells harboring Bcr-Abl or JAK2 constructs, as well as Ph+ patient-derived cell lines. Our data presented in Fig. 5a, demonstrated that the L isomer **(cpd 6)** exhibited a comparable activity against Ba/F3 cells carrying native and T315I mutated Bcr-Abl, and with a slightly enhanced potency against Ba/F3 cells carrying V617F mutated JAK2. In contrast, the D isomer **(cpd 7)** exhibited a comparable activity in inhibiting the clonigenicity of Ba/F3 cells harboring the mutated Bcr-Abl and JAK2 with reduced potency towards cells harboring the native Bcr-Abl in MEG-01 and K562 cells.

Although we have no experimental data showing the mechanism of action of the two oleyl amine derivatives, our docking experiments presented in Fig. 6 showed that a potential interaction in the Abl protein is located in the myristate-binding pocket (MBP), close to the region occupied by GNF-2. Furthermore, our molecular docking also argue that GNF-2, as well as the two oleyl amine derivatives, also interact with a hydrophobic region within the JAK2 protein, which resembles the myristate-binding pocket within the Abl protein.

Acknowledgments This work was supported, in part, by DFG-RU 728/3-2 to MR, YN and JM.

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